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The interests of the Laboratory of Biological Chemistry (LBC) cover a wide range of topics devoted to understanding biochemical and molecular events contributing to basic mechanisms of aging, as well as the development of age-related disabilities and diseases. The LBC is currently comprised of three major research units/section, the Molecular Neurobiology Unit (MNU), the Cell Biology Unit (CBU) and the Gene Expression and Aging Section (GEAS). A common goal of these programs is the elucidation of critical events associated with various age related deficits that could serve as targets for therapeutic strategies aimed at preventing or delaying the onset of disabilities and disease processes.

The Molecular Neurobiology Unit studies the structure, metabolism, and expression of factors controlling neuronal functions and their involvement in aging and age-related diseases. These factors include the amyloid precursor protein (APP) and presenilins, both of which are important in the pathogenesis of Alzheimer's disease, as well as glutamate receptors, neurotrophic factors, and extracellular matrix proteins.

The Cell Biology Unit encompasses studies on cancer and aging, physiological and molecular aspects of cartilage and bone function, and the role of mitochondrial dysfunction in aging and cell death. The group studying cancer and aging is interested in the role of vascularization in controlling the rate of tumor growth in the elderly, and molecular events important in the initiation and progression of breast cancer. The group studying cartilage is interested in examining the role of chondrocyte apoptosis in the etiology of osteoarthritis and in engineering chondrocytes in order to establish the feasibility of a homologous cell-based cartilage repair therapy in the elderly. Studies on bone involve assessing the causes of age-related deficits in old bone, and the design of new therapies for the treatment of osteoporosis. The group on mitochondrial dysfunction and aging is studying the contribution of mitochondrial DNA deletions to the aging process, and the role of mitochondrial dysfunction in causing programmed cell death. Studies in the Gene Expression and Aging Section

are focused on signal transduction pathways involved in regulating cellular responses to stress, the influence of these responses on growth regulation and homeostasis, and their alterations with aging.

While the individual research programs within the LBC generally function as independent groups, they are highly interactive, conduct biweekly joint meetings, and engage in collaborative projects. The Laboratory is equipped with state-of-the-art instrumentation and an extensive computer network.

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Recent Publications:

Gorospe M, et al. *Mol Cell Biol* 1996; 16: 6654-6660.

Guyton K, et al. *J Biol Chem* 1996; 271: 4138-4142.

Gorospe M, et al. *Oncogene* 1997; 14: 929-935.

Xu Q, et al. *J Clin Invest* 1996; 97: 508-514.

Biography: Dr. Holbrook received her Ph.D. from the University of South Florida, Tampa, Florida, in 1980. She completed postdoctoral training at Dartmouth Medical School and the National Cancer Institute. She moved to the NIA in 1986 to initiate a research program examining cellular responses to stress. She assumed the position of Chief of the Laboratory of Biological Chemistry in 1997.

Cellular Response to Stress and Aging: This research program focuses on cellular responses to stress and how they become altered with aging. The rationale for such studies is as follows: Aging is characterized by a general decline in most physiologic functions, and in particular, by a decreased capacity to maintain homeostasis during episodes of stress. These changes are believed to reflect the accumulation of damage to cells and tissues resulting from a variety of toxic factors, either produced endogenously during normal growth and metabolism, or derived from the environment. Normal function and survival are dependent on the cell's ability to resist or adapt to such stress and to repair or replace damaged molecules. Genetic systems have evolved to detect specific forms of damage and to activate the expression of genes whose products increase the resistance of the cell to damage or aid in its repair. The continued effectiveness of these genetic responses to environmental insults is likely to be a major factor in the resistance to disease and aging, and may be an important determinant of longevity.

Signal Transduction Pathways Mediating the Response to Genotoxic/ Oxidative Stress and Consequences for Cell Survival: A number of distinct pathways can be activated in response to stress, dependent on the nature of the insult. These include, but are not limited to, p53, the heat shock response, mitogen-activated protein kinase (MAPK) cascades, and NF*k*B. Although we have an interest in all of these pathways, much of our recent work has focused on the activation of the extracellular regulated kinase (ERK) and c-jun N-terminal kinase (JNK) MAPK cascades in response to oxidant injury. Efforts have concentrated on identifying the

initiating events and critical mediators involved in the response and determining the consequences of MAPK activation for cell survival. Through manipulation of the respective pathways we have shown that ERK activation is associated with enhanced survival, while JNK activation is associated with increased cell death following oxidant injury. Current studies are exploring downstream effectors of the ERK and JNK cascades, the interrelationships between MAPK cascades and other stress response pathways, and their role in *in vivo* models of stress.

Roles of Specific Stress-Induced Gene Products: More than 50 genotoxic stress-inducible genes have been identified in mammalian cells. Although the functions of many of these have not yet been identified, they are presumed to play an important role in determining cell fate. Depending on the particular stress or cell type examined, the response can range from proliferation and transformation, to transient or irreversible growth arrest, differentiation, or programmed cell death. Our research in this area examines the specific genes which are believed to mediate these differential effects. Our goal is to understand their regulation and determine their function during the stress response. Efforts over the past year have largely focused on the role of the cyclin-dependent kinase inhibitor p21/Waf1/Cip1 in mediating growth arrest and inhibiting apoptosis in various stress paradigms. Other gene products under study in this regard include the heat shock protein 70, p27/Kip1, and the growth arrest and DNA damage-inducible gene, GADD153.

Age-Related Alterations in the Stress Response: Aged cells and tissues exhibit a reduced ability to respond to environmental stresses. Studies in this project area are focused on identifying the causes for this altered responsiveness. We have recently demonstrated that aged hepatocytes show reduced activation of ERK in response to various stress stimuli including hydrogen peroxide, sodium arsenite, and heat shock. This results in reduced induction of ERK-regulated genes and is associated with reduced survival to arsenite treatment. Further studies will address which events lying upstream of ERK might be altered with aging, accounting for this change in responsiveness. Induction of heat shock proteins in response to heat is also reduced in aged cells. A long term goal is to devise strategies to up-regulate these stress responses in aged cells. Thus far, we have been successful in potentiating the heat shock response in aged hepatocytes using non-steroidal anti-inflammatory agents.

Collaborators: Robert Udelsman, M.D., Johns Hopkins University; John C. Lee, Smithkline Beecham Pharmaceuticals; Albert J. Fornace, Jr., NCI; George S. Roth, Laboratory of Cellular and Molecular Biology, NIA; Thomas W. Kensler, Johns Hopkins University; Prem Seth, NCI; Maurizio C. Capogrossi, M.D., Laboratory of Cardiovascular Science, NIA.

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Recent Publications:

Moyes C, et al. *Am J Physiol* 1997; 272: C1345-C1351.

Filburn CR, et al. *Mech Aging Dev* 1996; 87: 35-46.

Biography: Dr. Filburn received his Ph.D. at Purdue University and did postdoctoral training at Yale University studying regulation of cyclic nucleotide metabolism. He joined the NIA in 1973 as a Staff Fellow and continued studying hormonal regulation of cyclic nucleotide metabolism and action in the kidney and other systems. More recently he has focused his studies on the role of mitochondrial DNA damage and of mitochondrial dysfunction in cell death in aging and neurodegenerative disease.

Mitochondrial Deletions in Aging: A long-standing, still popular hypothesis about mechanisms of aging proposes that somatic mutations in mitochondrial DNA (mtDNA) increase with age and result in impaired energy metabolism in some, especially postmitotic, tissues. We have addressed this question in part by measuring deletions in animal models and in degenerating neuronal tissues of humans. Using PCR methodology we have shown that a 5.0 kb deletion in rat mtDNA increases markedly in liver and in specific regions of the brain over the 2 year lifespan of these animals. We found no association in the brain with loss of mitochondrial electron transport activity and increase in this deletion, possibly due to the fact that in no tissue do the levels of this or other mutations increase to the levels needed to impair energy metabolism. We have found a reduction in the buildup of this deletion in liver of rats placed on caloric restriction, a treatment that increases maximum lifespan and delays many of the effects of aging. Establishing the total mutational load due to deletions, duplications, and point mutations and determining whether this load actually causes impairment of mitochondria in old animals in a substantial way remains one of the major challenges in this field.

Mitochondrial Dysfunction and Apoptosis in Neuronal Cells: Impaired energy metabolism is known to sensitize neuronal cells to stresses, especially excitotoxins that can cause cell death. We are investigating the effects of reduced activity of components of the electron transport chain on cell survival using both rodent and human neuronal cell lines using specific inhibitors as well as cells harboring mitochondria with AD-associated mutations in cytochrome oxidase. In these studies we make extensive use of fluorescent techniques to assess *in situ* mitochondrial function, i.e., membrane potential and free radical production, as well as cell survival and death via apoptotic or necrotic processes. These studies also focus on the role of the mitochondrially-associated, antiapoptotic protein Bcl-X_L in protection against various insults and its mechanism of action. For example, we have shown that Bcl-X_L over expression in rat PC-12 cells protects cells from the death inducing effects of rotenone, a powerful mitochondrial inhibitor. More recently, we have begun studying the sensitivity to various stresses of human SH-SY5Y cells that have reduced cytochrome oxidase conferred to them by mitochondrial DNA from platelets of AD subjects. In addition, we are assessing the levels of AD-associated mutations in mtDNA-encoded cytochrome oxidase in white blood cells of subjects participating in the Baltimore Longitudinal Study of Aging. Lastly, we are overexpressing rat mitochondrial cyclophilin, a protein known to play a critical role in regulating mitochondrial membrane permeability in conditions of oxidative stress.

Collaborators: Richard Hansford, Ph.D., NIA; Wilhelm Bohr, Ph.D., NIA; John Kusiak, Ph.D., NIA; Bryan O'Connell, Ph.D., NIDR; Robert Davis, MitoKor, San Diego; Andrew Halestrp, University of Bristol, UK.

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Recent Publications:

Petersen E, et al. *Int J Imaging Syst Tech* 1997; 8: 285.

Zhao B, et al. *J of Neuroscience Research* 1997; 47: 253.

Horton WH Jr, et al. *Muscle & Nerve* 1998; 5: S79.

Horton WH Jr, et al. *In vitro Cellular & Developmental Biology* 1998; 43: 1.

Biography: Dr. Horton received his Ph.D. in Anatomy/Cell Biology from the University of Cincinnati. He carried out post-doctoral training in molecular biology at the University of North Carolina and the NIH. Dr. Horton worked at the Eli Lilly Company before moving to his present position in 1989.

Cartilage Biology: Models & Mechanisms Related to Aging & Disease: Cartilage undergoes degeneration with age resulting in osteoarthritis (OA). OA afflicts 60 million individuals in the U.S., most over the age of 60. The resident cells, chondrocytes, display genetic and biochemical alterations that likely contribute to disease progression. We are establishing model systems and studying mechanisms that contribute to these age-associated changes.

Mechanisms Contributing to Age-associated Changes of Cartilage: One dramatic change in cartilage with aging is a significant reduction in the number of chondrocytes. With time, this may result in too few cells to adequately replace the cartilage that is slowly degraded. We formulated a hypothesis that programmed cell death, or apoptosis might contribute to this loss of viable chondrocytes. We are the first group to provide direct evidence that articular chondroyctes die by apoptosis and that the incidence of apoptosis increases with age in animal models. We are now studying primary articular chondrocytes and various cell lines in culture to define the extracellular signals that initiate apoptosis and the intracellular molecules that mediate the signal to die. Our work on the role of apoptosis in the pathogenesis of degenerative diseases of aging such as OA may lead to a specific therapeutic target to prevent cell loss and maintain tissue function.

Evidence that age-associated OA has a genetic basis is limited. We have recently established that a polymorphic allele of the human aggrecan gene (which codes for the major proteoglycan of cartilage) is associated with individuals who have bilateral hand OA. This exciting finding supports the idea that a single gene defect may contribute to the pathogenesis of the most common form of OA.

Finally, the degeneration of cartilage is ultimately carried out by matrix metalloproteinases (MMPs). We have characterized a cell line (immortalized rat chondrocytes, IRC) that shows a pattern of MMP expression similar to what is observed in OA. Recently, we demonstrated that a particular porteinase (MMP-13) is up-regulated in IRC cells in response to cytokines. This cell line should be useful for studying the regulation of chondrocyte MMP expression and identifying inhibitors.

Regulation of Collagen II Gene Expression: Collagen II is the most abundant protein in cartilage and is expressd at high levels only by chondrocytes. We have identified sequences in the promoter and first intron of the collagen II gene that are important for its expression in chondrocytes and we are now characterizing the proteins that bind to these sequences.

Tissue Engineering: We are developing protocols and models to support tissue engineering therapies for cartilage disease. We are the first laboratory to demonstrate that chondrocytes isolated from the articular cartilage of mature animals and elderly OA patients can be induced to divide in culture. In addition, we have shown that these "secondary chondroprogenitor cells" will form hyaline cartilage *in vivo* and *in vitro*. The cells may be useful for the repair of degenerated cartilage in aged individuals.

Collaborative research has led to the development of a hollow fiber bioreactor (HFBR) that will support the 3-dimensional formation of cartilage from animal and human cells. The HFBR is compatible with analysis by nuclear magnetic resonance imaging and spectroscopy. This system will allow for the development of non-invasive methods to follow cartilage formation, maintenance, and response to growth factors and cytokines.

Collaborators: Dr. Karen Hasty, University of Tennessee; Dr. Kurt Doege, Shriner's Hospital; Dr. Marc Hochberg, University of Maryland; Dr. Jordan Tobin, Dr. Richard Spencer, and Dr. Antonino Passaniti, NIA.

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Recent Publications:

Wolozin B, et al. *Science* 1996; 274: 1710-1713.

Bai G, et al. *J Biol Chem* 1997; 272: 5936-5942.

Zhao B, et al. *J Neurosci Res* 1997; 47: 253-263.

Iwasaki K, et al. *Molec Psychiatry* 1996; 1: 65-71.

Biography: Dr. Kusiak received his Ph.D. from the Biochemistry Department of the George Washington University School of Medicine and Health Sciences in Washington, D.C. He did postdoctoral work in the Developmental and Metabolic Neurology Branch of the National Institute of Neurological Diseases and Stroke (NINDS), NIH before joining the Macromoleclar Chemistry Section of the Laboratory of Cellular and Molecular Biology, NIA. He spent a sabbatical year in the Receptor Biochemistry and Molecular Biology Section, NINDS. In 1990, he joined the newly formed Molecular Neurobiology Unit, Laboratory of Biological Chemistry, NIA where he has continued to study neurodegeneration in aging and diseases of aging.

Neurodegenerative Mechanisms in Aging and Alzheimer's Disease: Neurodegenerative diseases of aging including Alzheimer's and Parkinson's Diseases have distinct pathologies but both exhibit severe neuronal cell loss. The etiology of these diseases is obscure although excessive oxidative stress, environmental factors, and genetic factors have been proposed as initiating elements. Recent clinical studies of Alzheimer's disease (AD) patients treated with anti-inflammatory and anti-oxidant drugs suggest a potential ability of these drugs to slow the progression of the disease. One of the hallmarks of the disease is the presence in brains of extracellular senile plaques. A major constituent of senile plaques is the Aß peptide derived from a larger precursor protein, the Amyloid Precursor Protein (APP). Clues to the disease process come from recent discoveries of mutations in the APP gene and in two genes, unrelated to APP, termed Presenilins 1 and 2 (PS-1, PS-2). Mutations in these genes are found in early-onset, familial forms of AD and in each case lead to an increase in the production of longer forms (1-42) of the Aß peptide which has a greater tendency to aggregate and form senile plaques. *In vitro* studies showed that the Aß peptide is toxic to neuronal cells and the cell death induced by Aß may be apoptotic in nature.

Glutamate receptors play a pivotal role in several brain functions. However, over-activity of these receptors can lead to excitotoxic neuronal cell death. The type of cell death may be either necrotic or apoptotic depending upon the receptor subtypes involved and the degree of receptor stimulation. Interestingly, the distribution of these receptors correlates with the areas of cell loss found in AD. The receptors are important in learning and memory, processes severely impacted in AD, and over-activation of these receptors is thought to initiate a common final pathway of neuronal cell death in both acute and chronic brain insults.

Work in this group has focused on two areas of research: (1) the role of APP and PS genes in the pathology of Alzheimer's disease and (2) the transcriptional regulation of expression of the NMDAR1 gene, a key subunit of all NMDA receptors.

Amyloid Precursor Protein and Apoptosis in Alzheimer's Disease: A major focus of this project is to discover the roles of APP and the PS in the etiology and pathology of AD and the mechanisms involved in the neuronal cell death induced by mutant forms of these proteins. One of the aims of our laboratory is to discover how APP or PS mutations lead to specific neuronal cell loss in AD. Previously we showed that over-expression of mutated forms of APP in stably transfected PC12 cells led to the increased production of intracellular, amyloidogenic C-terminal fragments of APP. This is accompanied by increased cell death over several days; this death appears to be apoptotic by several criteria. Recently, we showed that transient expression of mutated forms of PS-2 also increased the amount of apoptosis in growth factor-dependent PC12 cells. In this same model system, the over-expression of an antisense PS-2 construct reduced the amount of apoptosis induced by mutant APPs suggesting that the two proteins may share the same pathway of cell death.

Taken together, these results suggest that the selective neuronal cell loss in AD may be due, in part, to an apoptotic mechanism. This provides a rationale for targeting particular elements of an apoptotic pathway for therapeutic intervention in AD. We are generating adenoviral vectors for injection into rat brains in order to examine the *in vivo* effects of over-expression of APP mutations. We will examine the effects of over-expression in specific brain regions and the possible differential sensitivity of older animals to an increased Aß load. We also are generating transgenic mice conditional for expression of mutated APPs in order to examine questions about the dynamics and reversibility of Aß deposition.

Transcriptional Regulation of NMDA Receptor Subunit Genes: A major focus of this project is to discover the pathological roles that excitatory amino acid (glutamate) receptors play in neuronal cell loss in aging and AD and the mechanisms by which this cell loss occurs. One of our objectives is to determine how the NMDAR1 and other family member genes are regulated at the transcriptional level. Since neurons expressing NMDA receptors are lost in AD, it may be important to determine which factors are involved in regulating expression and consequent activities of NMDA receptors during development and in aging and disease. Another objective of this project is to determine the mechanism by which glutamate causes cell death and the role activation of glutamate receptors plays in initiating a genetic cascade of programmed cell death. We previously characterized the promoter region of the NMDAR1 gene and found that it contained several transcriptional elements in the proximal region responsible for both basal, inducible, and neuronal specific expression. In recent work, we showed that nerve growth factor and other neurotrophins were able to stimulate expression of NMDAR1 gene in PC12 cells. This stimulation was induced through activation of high affinity TrkA receptors and subsequent activation of a Ras/Raf MAP kinase pathway. In preliminary results, we showed that this activation may be mediated through both GSG and Sp1 transcription factor activation and that Sp1 may be a novel phosphorylation target of Erk. In other work, we characterized several promoters for the second family of NMDA receptor subunits and found that they share some similar characteristics with the NR1 promoter. Other elements in the NMDAR1 promoter are being studied including two CRE consensus sites in the proximal region which may have important roles in regulating expression.

We are examining several neuronal cell lines as potential models to study glutamate-induced excitatory neurotoxicity. We showed that glutamate treatment of a hippocampal cell line will cause cell death which can be prevented by the over-expression of Bcl-2 protein. The results suggest that this line may be a good model to study neuronal apoptosis. NT2-N cells express the NMDA subtype of receptor and may be a good model of glutamate-induced necrotic cell death.

Collaborators: Sangram Sisodia, Ph.D., Johns Hopkins University; Benjamin Wolozin, M.D., Loyola University; Andres Buonanno, Ph.D. and Mike Sasner, Ph.D., Laboratory of Developmental Neurobiology, NICHD; Lin Mei, M.D., Ph.D., University of Virginia; Stuart Lipton, M.D., Harvard University; Eva Eves, Ph.D., University of Chicago.

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Recent Publications:

Tanaka H, et al. *Bone* 1996; 18: 473-478.

Williams S, et al. *Bone* 1996; 19: 637-644.

Tanaka H, et al. *Mech Ageing Develop* 1996; 92: 1-10.

Biography: Dr. Liang received his Ph.D. from Kansas State University, Manhattan, Kansas, in 1972. After two years postdoctoral training at Duke University, he joined the NIA in 1974. He is a member of the American Physiological Society and the American Society of Bone and Mineral Research.

Bone Biology and Aging: Our main interests are to define the causes for age-associated deficits in bone remodeling activity and to develop novel treatment approaches for osteoporosis. In the past year, we have continued to focus on three areas.

Effect of Matrix Proteins on Characteristics of Osteoprogenitor Cells: The expression of extracellular matrix proteins declines in old bones and consequently, may lead to impaired development and function of old bone cells. To test this hypothesis, we examined the growth of osteoprogenitor cells in dishes coated with matrix proteins. We have shown that the number of osteoprogenitor cells is stimulated 50%, 30% and 15%, respectively, in culture dishes coated with laminin, type I collagen or fibronectin. Type IV collagen has no effect on osteoprogenitor cells. Increase in cell number can be attributed to the increase in colony number not the size of colonies. Since matrix proteins may also affect the osteogenic lineage of progenitor cells, we examined the number of colonies that express alkaline phosphatase, an indicator of osteogenic linage commitment. We found that the alkaline phosphatase positive colonies were reduced 35% and 25%, respectively, by laminin and type I collagen. However, these effects were the same whether cells are derived from adult or old bones. To assess the possibility that different matrix proteins may also affect lineage development of osteoprogenitor cells in culture, we determined the bone induction potential of cells grown in dishes coated with matrix protein. Bone forming activity of old osteoprogenitor cells in subcutaneous implants is approximately 1/6 that of adult progenitor cells. However, culturing of osteoprogenitor cells on matrix proteins has no apparent effect on bone induction potential regardless of the age of animals from which the cells are derived. We have

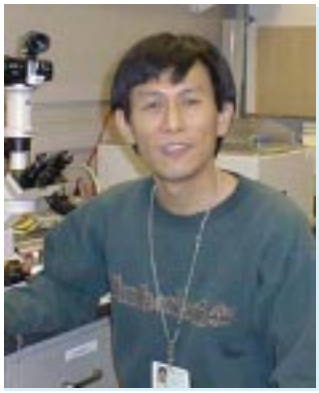
concluded that the decline in the expression of matrix proteins in senescence may impair the growth of osteoprogenitor cells but not the bone induction potential of old progenitor cells.

Normalizing Bone Turnover Activity with Growth Factors: Previously, we have shown that locally infused IGF-I can stimulate the expression of matrix proteins and increase trabecular bone volume in old femurs. In the past year, we examined the bone remodeling activity in old femurs after IGF-I treatment (50 ng/day, 14 days). We have observed that IGF-I treatment increases trabecular volume, trabecular number, and trabecular thickness, and decreases trabecular separation. IGF-I infusion increases the number of osteoblasts and osteoblast surface without significantly affecting the number of osteoclasts or osteoclast surface. Other parameters that assess the organic matrix (osteoid surface, osteoid volume) are also increased by IGF-I. Kinetic indices associated with bone formation, mineralizing surface, mineral apposition rate and bone formation rate, are elevated in IGF-I-treated femurs. Eroded surface, a resorption index, is not affected. We conclude that IGF-I treatment can improve trabecular bone status in old rats and that this effect is solely the result in changes in bone formation. Interestingly, it has been shown in clinical studies that subcutaneous injection of IGF-I at a low dose in elderly women can elevate serum markers of bone formation. At higher doses, IGF-I increases markers for both bone formation and resorption. The serum levels of IGF-I in research subjects treated with a low dose of IGF-I are elevated 100% which is similar to the estimated increase of IGF-I content in the marrow cavity in animals infused with 50 ng/ml of IGF-I.

Phase II Clinical Trial with Minocycline in Treatment of Postmenopausal Osteoporosis: We have shown that minocycline can prevent the loss of bone mass and trabecular bone in estrogen-deficient old rats by stimulating bone formation and, concurrently, inhibiting bone resorption. We have initiated a phase II clinical trial using minocycline to treat postmenopausal osteoporosis. Currently, human subjects with bone mineral density (either in the spine or hip) at 2.5-3.5 SD below the peak levels of young adults are being recruited for the study. Of fourteen subjects who passed the preliminary evaluation, four fit our criteria in further testing and were admitted to our clinical trial program. One subject developed an unrelated complication and was removed from the program. The treatment period will be for one year with 200 mg of minocycline by mouth given daily. Bone mineral density will be determined before the treatment, at 6-months, 12-months during the treatment and 4-months after the treatment. Serum and urine will be collected at 4-month intervals and markers of bone formation and resorption will be assayed during the treatment.

In the coming year, we are planning to expand our effort to recruit patients to the clinical trial with minocycline. In a parallel study, we will also examine the effect of minocycline on restoring the low bone mass in chronically estrogen-deficient old rats. Changes in femoral gene expression and alterations in bone turnover activity will be assessed to elucidate the mechanism of minocycline action.

Collaborator: Jay Shapiro, M.D., Johns Hopkins Bayview Medical Center.



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Recent Publications:

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Liu Y, et al. *J Biol Chem* 1996; 271: 3604-3607.

Liu Y, et al. *Free Radic Biol Med* 1996; 21: 771-781.

Biography: Dr. Yusen Liu received his Ph.D. in molecular biology and yeast genetics in 1991 from the Department of Fermentation Technology, Hiroshima University in Japan. He served briefly as an assistant professor in the same department, before joining NIA's Gene Expression and Aging Section in 1992, as a Visiting Fellow. In 1995 he was promoted to the position of Visiting Associate, and in 1996 to the position of Investigator. Dr. Liu's research is on signal transduction pathways involved in the stress response and their implications to the aging process.

Signal Transduction Pathways Involved in the Stress Response and Aging: Exposure of eukaryotic cells to harmful environmental conditions evokes alterations in gene expression. In mammalian cells, the stressful signals are mainly sensed by the cell membrane and transduced through posttranslational modifications of key regulatory molecules. Almost immediately after exposing cells to genotoxic agents, increases in the activities of a number of protein kinases can be detected. Activation of these primary protein kinases initiates the protein phosphorylation cascades, leading to the activation of a group of mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase/Stress-activated protein kinase (JNK/SAPK) and the p38 MAP kinase. These MAP kinases can phosphorylate and activate

downstream protein kinases and a variety of transcription factors including Elk-1/TCF, c-Jun, ATF-2, and c-Myc, resulting in changes in gene expression. Altered gene expression can, at least in part, account for the variable phenotypical changes cells undergo after stress. Thus, in order to understand the molecular basis for the diversity in gene expression as well as phenotypical cellular outcomes, it is critical to understand the signal transduction pathways involved in the stress response and the transcription factors activated by these pathways.

Work in this group is aimed at understanding the molecular basis for the activation of MAP kinases in response to stress, and identifying the downstream targets regulated by them.

We have previously demonstrated that stressful stimuli can differentially activate ERK, JNK and p38 MAP kinases. Using sodium arsenite as a model for chemical stress, we have shown that in PC12 cells the activation of ERK is dependent on the activity of the proto-oncoprotein Ras, while activation of JNK and p38 does not require Ras activity. Similarly, activation of ERK is sensitive to pretreatment with the growth factor receptor blocker suramin, consistent with the model that ERK activation by arsenite involves activation of growth factor receptor tyrosine kinases. The fact that suramin has little effect on the activation of JNK and p38 suggests that growth factor receptor tyrosine kinases are not involved in their activation by arsenite. Recently, we extended our studies to signals downstream of ERK, and demonstrated that arsenite also activates the ERK-regulated protein kinase $p90^{RSK}$, a downstream serine/threonine kinase also known to be involved in transcriptional control. The $p90^{RSK}$ activation follows activation of ERK, and is correlated with the phosphorylation and degradation of the NF-κß inhibitor, Iκß , suggesting that it may play a role in regulating the activity of the transcription factor NF-κß . Supporting our hypothesis, very recently $p90^{RSK}$ has been demonstrated by others to be a physiological regulator of Iκß . We are currently testing the role of $p90^{RSK}$ in the activation of NF-κß in response to arsenite.

Since Ras plays a crucial role in activating ERK in response to arsenite and a growth factor receptor may be involved, it is likely that the adaptor protein Grb2 is involved in the activation pathway. Consistent with this, we found that arsenite strongly stimulates tyrosine phosphorylation of Shc and its interaction with Grb2. Furthermore, we have recently been able to co-immunoprecipitate a high molecular weight protein that is also tyrosine phosphorylated with kinetics similar to those of Shc. The identity of the high-molecular weight protein is currently unclear, but it may belong to the growth factor receptor tyrosine kinase family. We hypothesize that

arsenite activates a receptor tyrosine kinase through a mechanism independent of growth factors, resulting in its autophosphorylation on tyrosine residues. Shc binds to the phosphotyrosine residue through its SH2 domain, and itself becomes tyrosine phosphorylated. The phosphotyrosine residues then recruiting provide docking sites for Grb2 to the membrane, locating the Ras activator mSos to its target-membrane-associated Ras, leading to the activation of the ERK MAP kinase cascade. Currently, we are testing our hypothesis.

In future studies we plan to further establish the role of Shc and Grb2 in stress-induced ERK activation. We would also like to understand the identity of the tyrosine-phosphorylated high-molecular weight protein interacting with Shc. As a long-term goal of this program, we would like to establish a screening system to identify genes encoding substrates of JNK and p38. These should provide further insight into the functions of these pathways.

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Cirielli C, et al. *J Neuro-Oncology* 1997; 31: 217-223.

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Jiang B-H, et al. *Cancer Res* 1997; 57: 5328-5335.

Pili R, et al. *Int J Cancer* 1997; 73: 258-263.

Biography: Dr. Passaniti received his Ph.D. at the University of Virginia and completed his post-doctoral training in cell biology at the University of Maryland and in tumor metastasis at the Johns Hopkins University. He joined NIA's Laboratory of Biological Chemistry in 1989 as a Staff Fellow and expanded on his work in tumor biology which now includes the investigation of anti-angiogenic therapeutics and mechanisms of breast cancer progression.

The Biology of Cancer and Aging: The goal of our studies is to understand the events and to elucidate the mechanisms contributing to age-related changes in tumor growth, progression, and angiogenesis. Using transplantable murine models to study tumor growth and angiogenesis, we have found significant age-related deficits in vascularization and are using an unique *in vivo* angiogenesis assay to evaluate angiogenic and anti-angiogenic factors. Our approach to study tumor progression is to determine the genetic and epigenetic events that control the loss of hormone responsiveness in a rat model of spontaneous breast cancer.

Tumor Growth and Vascularization: We are investigating several aspects of tumor vascularization: (1) inhibitors of angiogenesis that are isolated from tumor matrix, (2) tumor stromal cells and their role in tumor growth, (3) mechanisms of endothelial cell death, and (4) testing of angiogenic and anti-angiogenic agents using an *in vivo* assay.

Inhibitors of endothelial cell proliferation were found in extracellular matrix isolated from tumors grown in aged mice. These studies led us to design cell differentiation and apoptosis assays to isolate these factors. In collaboration with colleagues at our institute, we are also using chondrocyte differentiation assays to identify these factors. These factors may be important determinants of cellular differentiation and in the observed slower growth of tumors in aged mice. It is commonly believed that tumor-infiltrating cells contribute to the vascular response and to tumor cell growth. We have been isolating stromal cells from tumors

grown in animals of different ages and are now testing these cells for their biological activity using vascular and tumor cells as targets.

We have found that endothelial cell apoptosis is mediated by protein tyrosine phosphatases. Using fluorescent Tunel labeling to detect DNA fragmentation *in situ,* DNA laddering, and expression of the apoptosis-specific gene, TRPM-2, morphological changes diagnostic for apoptosis were correlated with the loss of tyrosine phosphorylation. Focal adhesion kinase, MAP kinase and cdc2 expression and phosphorylation were also examined. Actin filaments were found to depolymerize at the onset of apoptosis, an effect that could be prevented by inhibition of protein tyrosine phosphatases. We found that expression of the cyclin-dependent kinase inhibitor p21 was associated with survival of endothelial cells. Current efforts are examining the role of the bcl-2 family genes in endothelial cell apoptosis and how the expression of NFkB is related to phosphatase activation.

Using an *in vivo* angiogenesis assay, which we developed to investigate the response of aged mice to different angiogenic factors, we are testing novel chemotherapeutic drugs for anti-angiogenic (and therefore anti-tumor) activity. The use of angiogenic and anti-angiogenic adenoviral vectors in this assay has allowed us to study the role of VEGF, FGF, and the tumor suppressor gene p53 in tumor growth, vessel restenosis, and vascularization. This assay has also been useful for screening compounds that enhance angiogenesis, an application of particular interest in the aged because of the increased incidence of chronic wounds and diabetes.

Breast Tumor Progression: We have established a tumor progression model in aged rats in which estrogen receptor positive cells (ER+) become ER negative (ER-). We have observed this progression *in vitro* and *in vivo*. Using somatic cell fusion, we obtained hybrid clones that were predominantly ER+. The dominance of the ER+ phenotype implies that the ER+ fusion partner is contributing something missing from the ER- partner. These data suggest that a possible loss of tumor suppressor genes may regulate the progression. In these studies we have found that, unlike ER- cells, ER+ cells express more keratin and less vimentin, express desmosomal (junction) proteins, and respond to hormones and growth factors, including estradiol, dexamethasone, insulin, progesterone, and EGF. We are continuing studies to clone possible tumor suppressor genes and have completed our immediate goals by making a cDNA library from ER+ cells and investigating further phenotypic differences in these cells to allow the screening of suppressor genes. We have found that ER- cells are more invasive than ER+ cells and activate metalloproteinases. The ER+ cells are more sensitive to apoptosis than are ER- cells and we are looking at the mechanisms of this change. In addition, we are using transfection of

ER- cells with ER+ cell cDNA vectors and screening for the acquisition of specific proteolytic expression to identify putative tumor suppressor genes.

We have also analyzed the methylation status of the ER region encompassing exon 1 of the ER gene in primary rat mammary tumors to see if this is involved in tumor promotion in the aged rat. Our findings indicate that lower methylation is associated with increased tumor incidence. Hypomethylation occurs even in tumor-free mammary glands in aged rats, but not in middle-aged or young rats. Further studies in young rats induced with the mutagen DMBA have also shown lower methylation at this locus, even in normal glands, before tumor onset. Hypomethylation of the ER gene may, therefore, be a common event in mammary tumorigenesis in the rat and may be of predictive value as a marker of increased breast cancer risk. Continuing studies are aimed at investigating the role of dietary fat and calories in the regulation of ER methylation.

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Isakov N, et al. *J Biol Chem* 1996; 270: 15753-15761.

Wange RL, et al. *Immunity* 1996; 5: 197-205.

Biography: Dr. Wange received his Ph.D. from the Department of Pharmacology at Vanderbilt University in 1991. He received his postdoctoral training at the Cell Biology and Metabolism Branch of the National Institute of Child Health and Human Development (NICHD) before becoming an Investigator in the Gene Expression and Aging Section of the Laboratory of Biological Chemistry in 1997. His research focuses on the signaling pathways involved in T lymphocyte activation.

Aging and T Lymphocyte Activation: A hallmark of aging in higher animals is a general decline in immune function. This is seen both as a decrease in the robustness of certain protective immune responses, and an increase in the occurrence of auto-immune responses. It has even been proposed that increased immune self-reactivity may play a causative role in aging, rather than being just another symptom of aging. It is our contention that a better understanding of the mechanisms by which the immune system becomes dysfunctional with age will enable the development of pharmacological interventions that will restore immune function, and perhaps alleviate some of the morbidity associated with aging.

T lymphocytes are vitally important in mounting an effective immune response. They not only orchestrate the immune response by regulating the activity of other components of the immune system, but also are directly involved in the surveillance and killing of infected or cancerous cells. Given the importance of T cells in initiating and maintaining the adaptive immune response, many of the studies aimed at understanding the defect underlying the decline in immune function that accompanies aging have focused on T cells. The fraction of T cells that are responsive to mitogenic stimuli declines with age. When compared to T cells from young animals, these unresponsive T cells exhibit aberrant biochemical responses to mitogens, including weak mobilization of intracellular calcium and aberrant phosphorylation of intracellular signaling proteins. This suggests that there are significant changes in the signal transduction pathways that

occur in T cells with aging. The goal of this research program is to characterize the signaling pathways that transduce proliferative signals received at the plasma membrane of T cells into the coordinated responses of proliferation and acquisition of effector function. We also want to identify which components of this pathway become altered with aging, and to understand the mechanisms that underlie these changes.

T Cell Receptor Signaling: In order to understand the nature of the signaling defects in T lymphocytes from aged animals, one must first understand the signal transduction pathways used by normal T cells. With this in mind, the laboratory is studying the molecules involved in normal TCR signaling. A key TCR-associated signaling molecule is the protein tyrosine kinase ZAP-70, which is required in order to signal through the TCR. Understanding the mechanism of action of ZAP-70 and how its activity is regulated is one of the priorities of this program. Precisely how ZAP-70 regulates T-cell proliferation is incompletely understood. We know that upon stimulation of the TCR that ZAP-70 is recruited to tyrosine-phosphorylated motifs within the TCR, whereupon ZAP-70 is tyrosine phosphorylated and activated by a heterologous kinase. But we know little else, except that there is no signaling through the TCR in the absence of ZAP-70. Even the substrates of ZAP-70 largely remain to be identified. One interesting question concerns the location of ZAP-70 before its recruitment to the TCR and whether or not release from this site is a regulated event. Although current models contend that ZAP-70 is freely cytosolic, observations in our laboratory and in others suggest that ZAP-70 may actually be associated with the cortical cytoskeleton in unstimulated T cells. Efforts are underway in the lab to assess this possibility. In addition we are using 2-hybrid and tribrid genetic screens to identify additional ZAP-70-associated proteins, which may play a further role in regulating or mediating ZAP-70 activity.

With advancing age there is also a general decline in the ability of cells to cope with oxidative stress. T cells are particularly susceptible to changes in oxidative tone, and there is some evidence of a redox sensitive signaling component to the TCR signaling pathway. In fact, oxidative stressors such as H2O2 or UV have been used to mimic the effect of TCR engagement, since many of the biochemical events initiated by TCR engagement can also be initiated by oxidative stress. The effects of these agents have been shown to be dependent on the presence of an intact TCR. Given the importance of ZAP-70 in mediating TCR signals, the role of ZAP-70 in propagating oxidative signals in T cells also needs to be assessed. We are currently using a ZAP-70 negative Jurkat T cell line to access the role of ZAP-70 in oxidative signaling. Preliminary results support a role for ZAP-70 in mediating these oxidative signals. Whether ZAP-70 localization or

activity is altered with age remains to be established, as does the physiological significance of the sensitivity of ZAP-70 activity to oxidative stimuli. These questions will be the subject of future investigations.

CD28 Signaling: T cell proliferative responses require engagement of a co-stimulatory receptor, such as CD28, in addition to TCR engagement. Stimulation of CD28 results in increased tyrosine phosphorylation of several substrates, as well as activation of phosphoinositide 3-kinase, sphingomyelinase and perhaps other enzymatic activities, ultimately causing the activation of transcription factors of the rel family. The precise signaling pathways that link CD28 engagement with transcriptional activation remain unclear, as does any role for CD28 in mediating the effect of aging on T-cell function. The lab is initiating a program that will induce somatic mutations within the pathways linking CD28 engagement and transcriptional activation. This project has the potential to identify signaling molecules previously unrecognized as playing a role in CD28 signaling, and will also provide null cells in which the functional elements of these unknown and known signaling molecules can be studied.

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